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CMM COLOGNE

Dystrophin Quantification in Clinical Trials

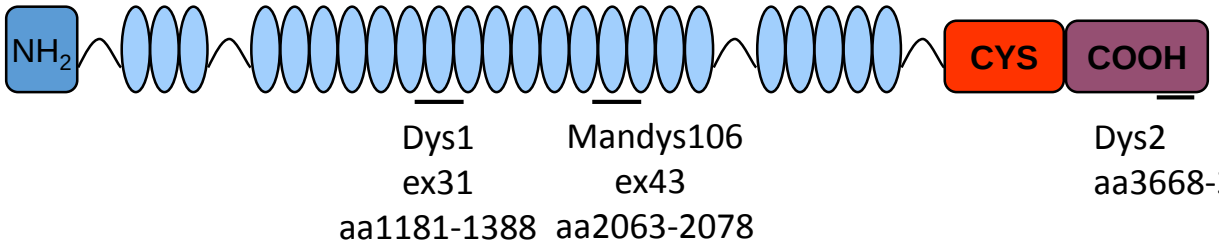
Sebahattin Cirak

University Hospital Cologne, Germany

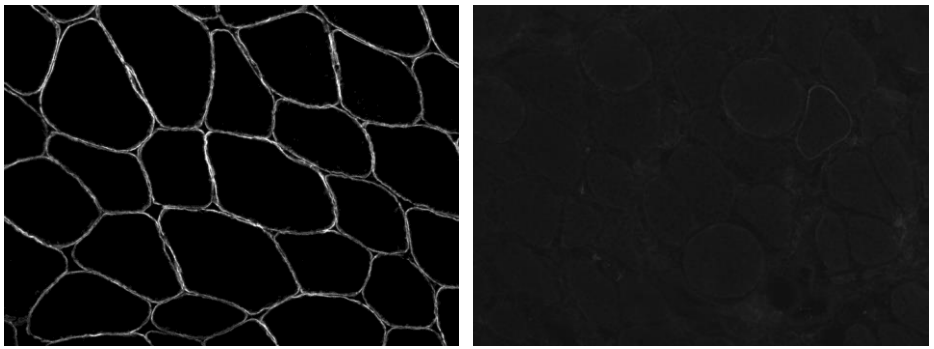
Dystrophin detection

- Dystrophin quantification methods were developed from routine diagnostic tests for Duchenne Muscular Dystrophy

Dystrophin protein



Dys 2



Control DMD

- Usually, immunofluorescence staining of cryosections with monoclonal dystrophin antibodies
- Western Blots developed further in particular for Becker Muscular Dystrophy Patients
- Later need to distinguish between revertant fibers and new restored dystrophin after exon skipping

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AVI-4658 intra-muscular study NCT00159250

Lancet Neurology 2009

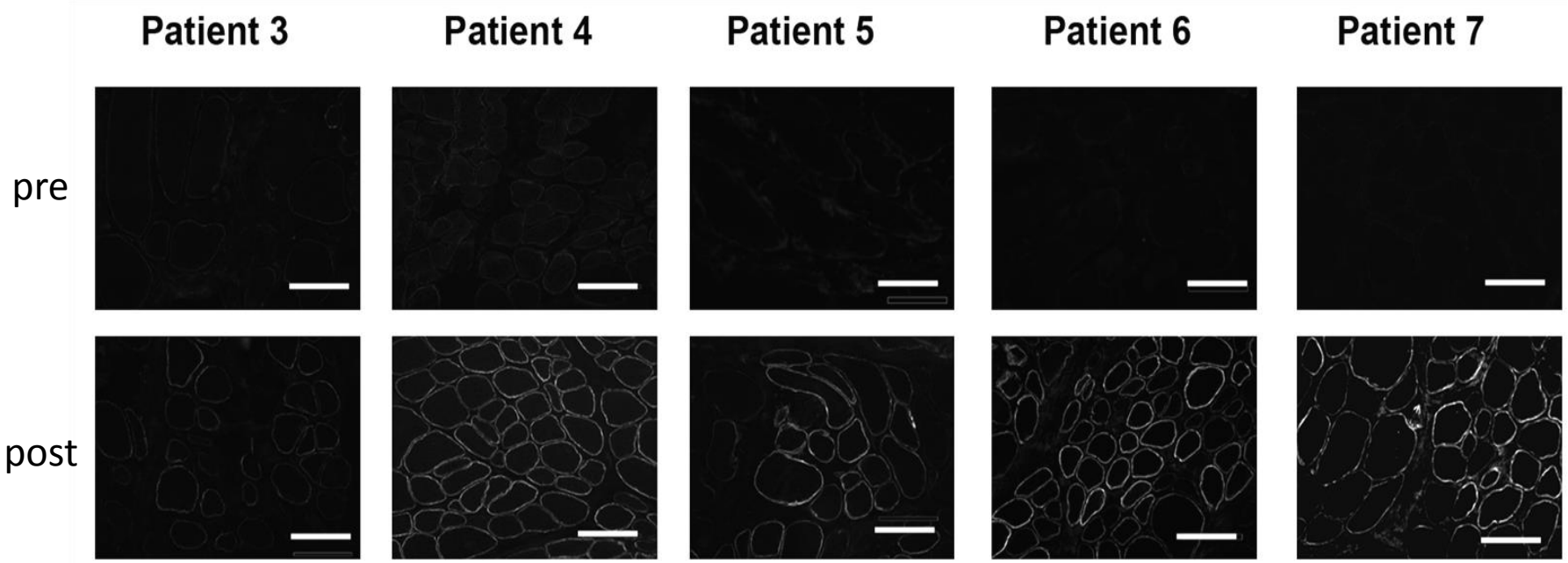
UCL MDEX

Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study

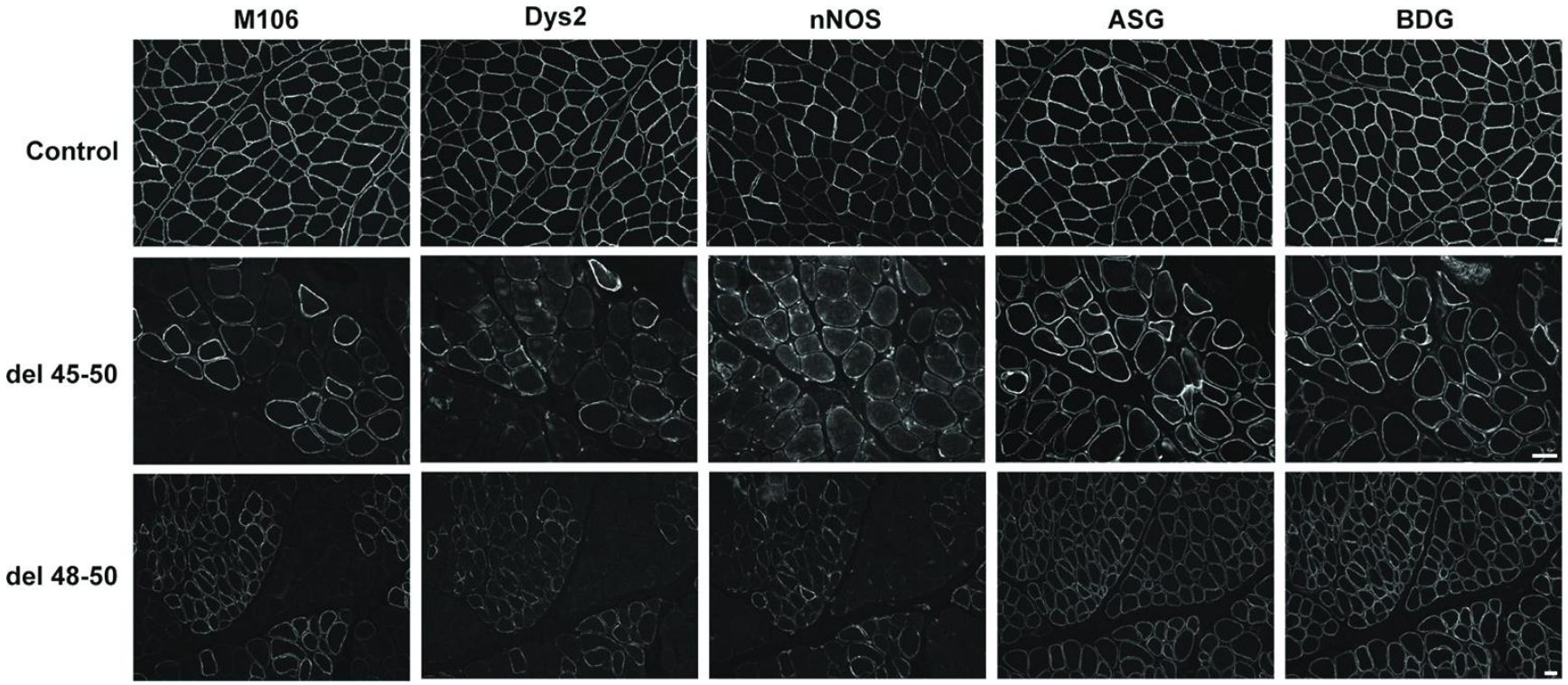
Maria Kinali*, Virginia Arechavala-Gomeza*, Lucy Feng, Sebahattin Cirak, David Hunt, Carl Adkin, Michela Guglieri, Emma Ashton, Stephen Abbs, Petros Nihoyannopoulos, Maria Elena Garralda, Mary Rutherford, Caroline Mcculley, Linda Popplewell, Ian R Graham, George Dickson, Matthew JA Wood, Dominic J Wells, Steve D Wilton, Ryszard Kole, Volker Straub, Kate Bushby, Caroline Sewry, Jennifer E Morgan, Francesco Muntoni

Intra muscular dose EDB muscle
5 boys high dose (0.9 mg in 900 µL)
EMG needle injection

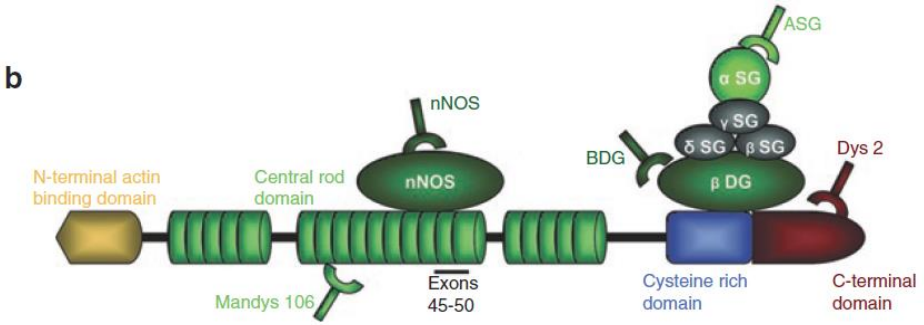
Mandys106 AB 1:100



Restoration of the DGC and deletion specific restoration of nNOS



Cirak et al., 2012



Cirak et al., 2012

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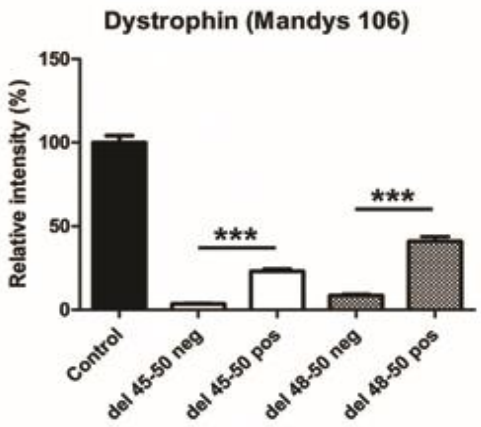
Quantification by Immunofluorescence confirmed increase of DGC proteins after treatment.

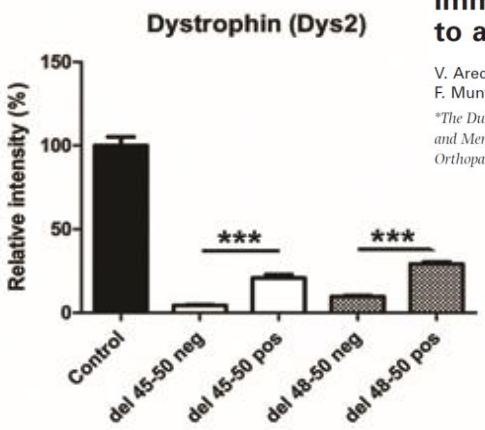
Neuropathology and Applied Neurobiology (2010), **36**, 265–274 doi: 10.1111/j.1365-2990.2009.01056.x

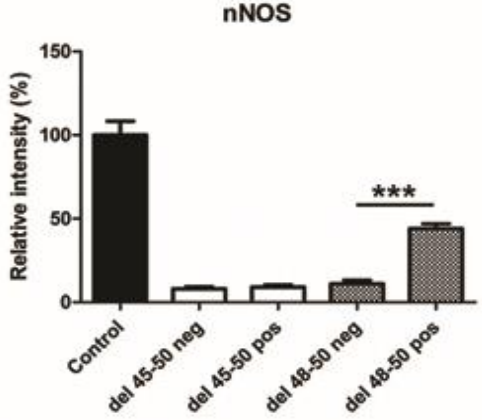
Immunohistological intensity measurements as a tool to assess sarcolemma-associated protein expression

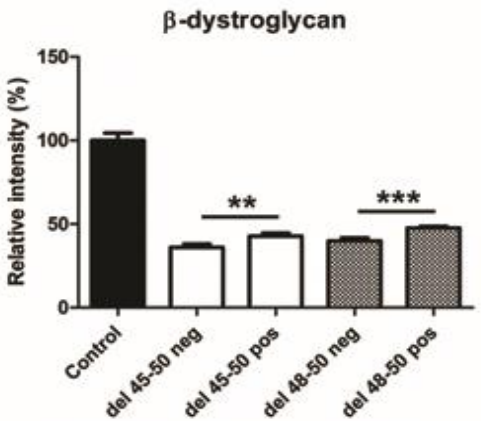
V. Arechavala-Gomeza*, M. Kinali*, L. Feng*, S. C. Brown†, C. Sewry‡, J. E. Morgan* and F. Muntoni*

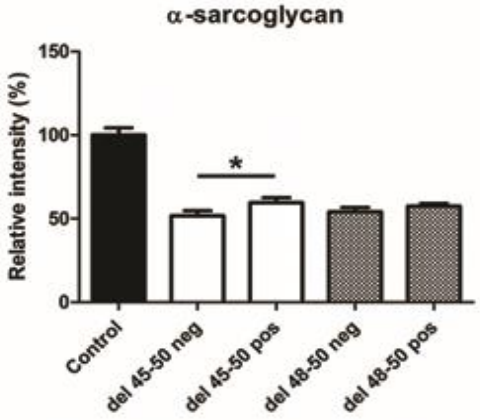
**The Dubowitz Neuromuscular Centre, Institute of Child Health, University College London, †Division of Neurosciences and Mental Health, Imperial College London, London, and ‡Centre for Inherited Neuromuscular Diseases, RJAH Orthopaedic Hospital, Oswestry, UK*











Cirak et al., 2012

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Systemic AVI-4658-28 Systemic study

UCL MDEX

	Study ID	Deletion	Age at first dose (years)	Number of doses	Cumulative dose, during 12 weeks' treatment (mg)
Cohort 6 (20 mg/kg)	P19	45–50	9	11	6710
	P18	49–50	10	12	10788
	P17	45–50	7	12	5673
	P16	45–50	9	12	6982
Cohort 5 (10 mg/kg)	P15	49–50	10	12	4866
	P14	47–50	12	12	6207
	P13	48–50	7	12	2664
	P12	49–50	6	12	3036
Cohort 4 (4 mg/kg)	P11	45–50	9	12	1342
	P10	48–50	10	11	2801
	P9*	52	10	7	864
Cohort 3 (2 mg/kg)	P8	49–50	9	12	924
	P7	49–50	13	12	1113
Cohort 2 (1 mg/kg)	P6	48–50	6	12	255
	P5	45–50	6	12	326
Cohort 1 (0·5 mg/kg)	P4	48–50	8	10	180
	P3	49–50	8	12	218
	P2	45–50	8	12	171
	P1	48–50	9	12	186

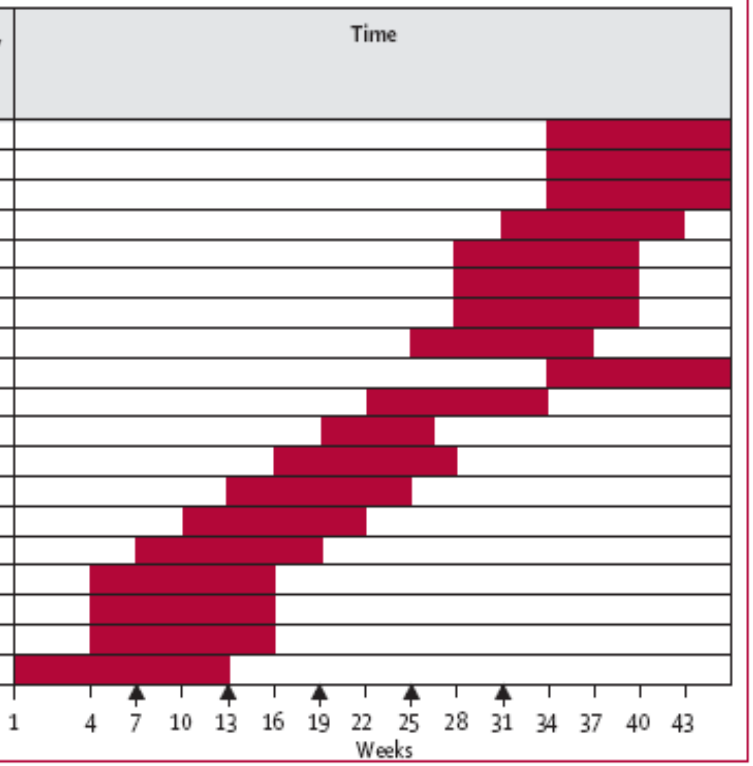

Figure 1: Patients recruited to the trial, their assignment to cohorts, and the dose-escalation scheme
Each full red box represents a time interval of 12 weeks' dosing. Arrows show the timepoints at which the data safety monitoring board met with clinical investigators and the sponsor to review safety before subsequent dose escalations. *Patient withdrawn from study after seven doses.

- **6 Cohorts Dose Escalation**
- **Open-label, no randomization**
- **1 h intravenous infusion**
- **Weekly for 12 weeks**

- **Further F/U atweeks 18, 22 and 26**
- **Each patient had a baseline muscle biopsy before recruitment and 2 weeks after last dose**

Cirak et al., 2011

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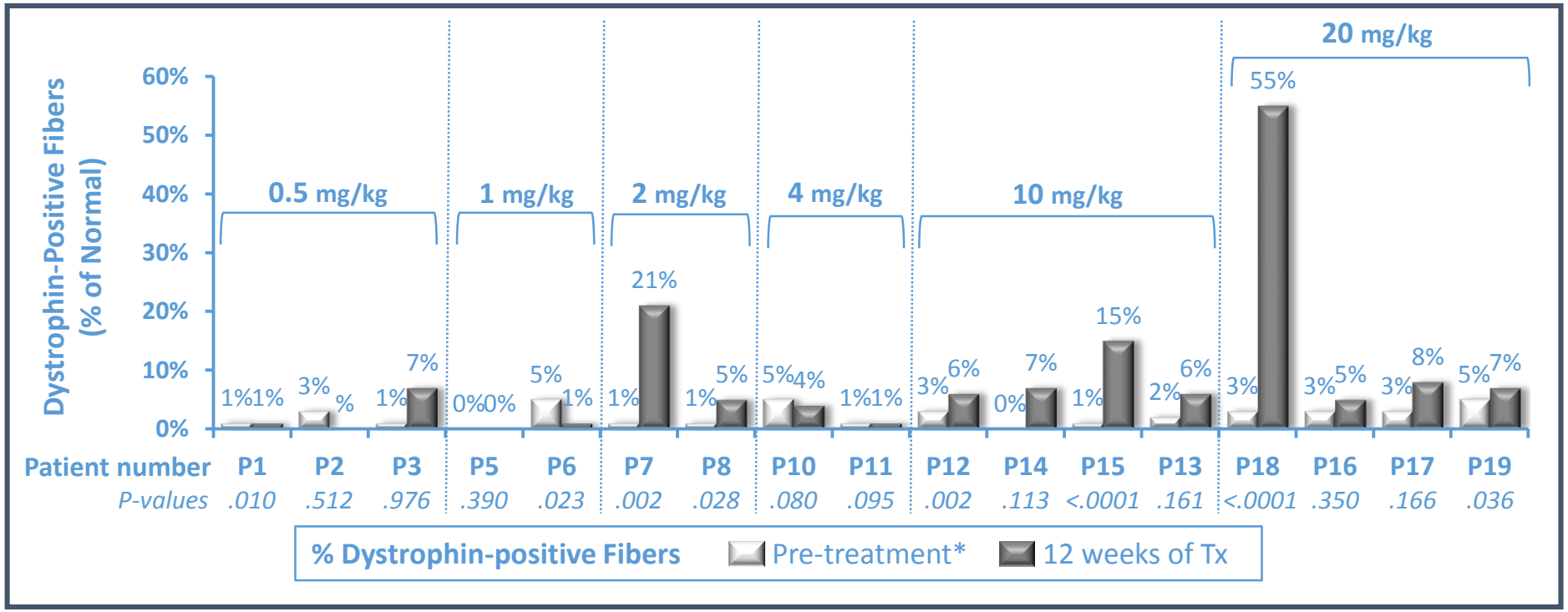
Dystrophin positive fibers

Method: Cryosections 8 µm, Mandys106 (exon 43) 1 h incubation, washed with PBS, biotinylated secondary anti-mouse 1:200 for 30 min, washed in PBS and developed Alexa 594 (1:2000) for 15 min. PBS washed and mounted with Hydromount.

Assessment: Initial assessment by blinded investigators, then adjusting the threshold for each subject so that only revertant fibers were detected in the pre-treatment biopsy.



Pre-treatment samples were used as a baseline to count dystrophin-positive fibres in post-treatment muscle.
Cirak S, et al. *Lancet.* 2011;378(9791):595-605.

MDEX

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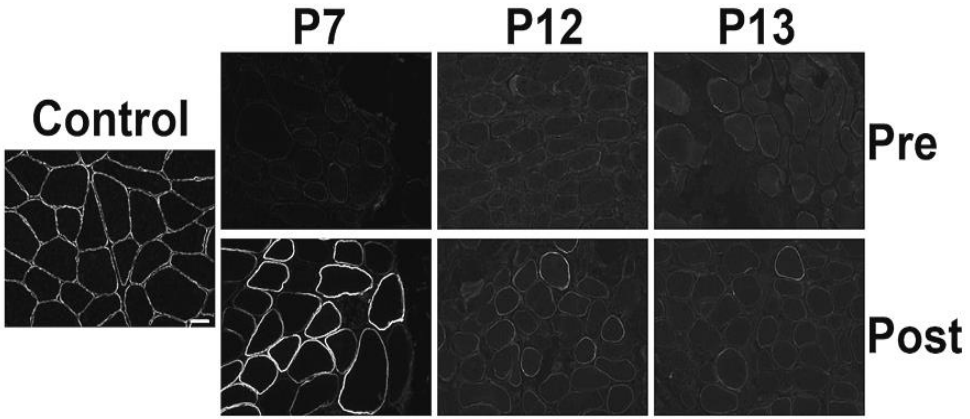
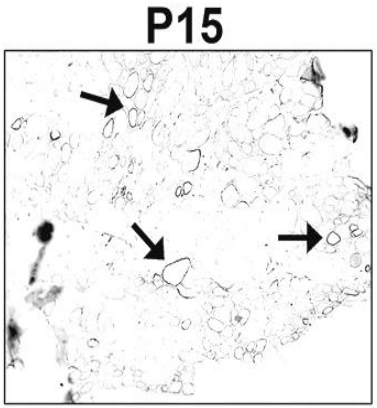
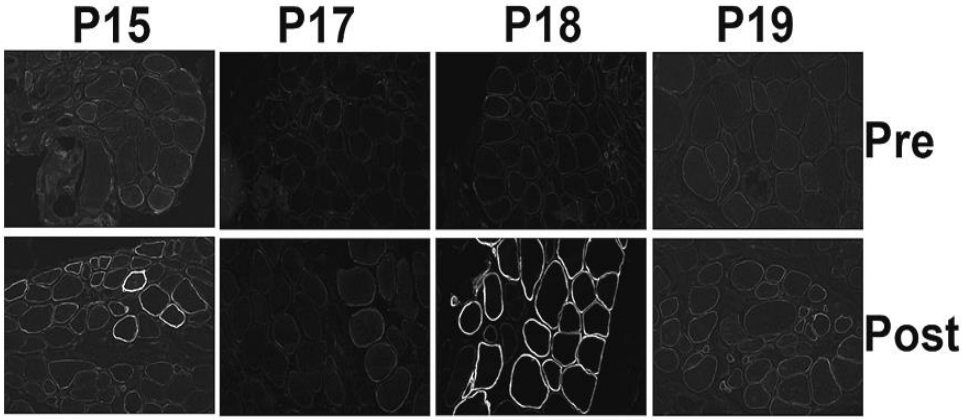
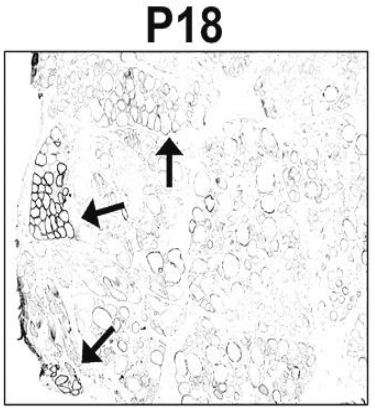
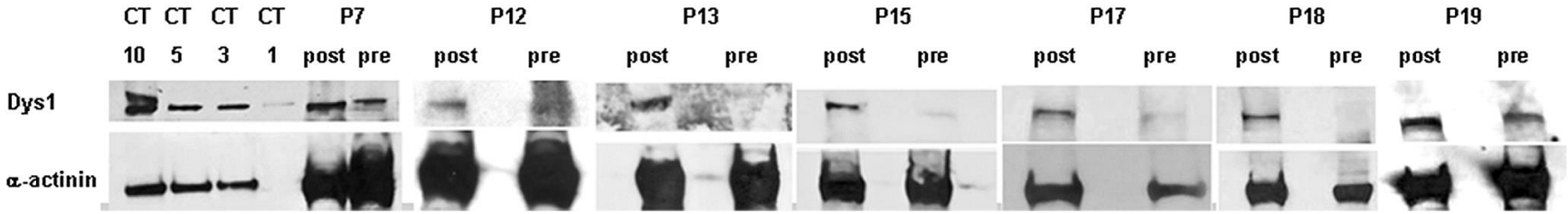
Dose response in dystrophin expression, AVI-4568-28 study











Cirak et al., 2011

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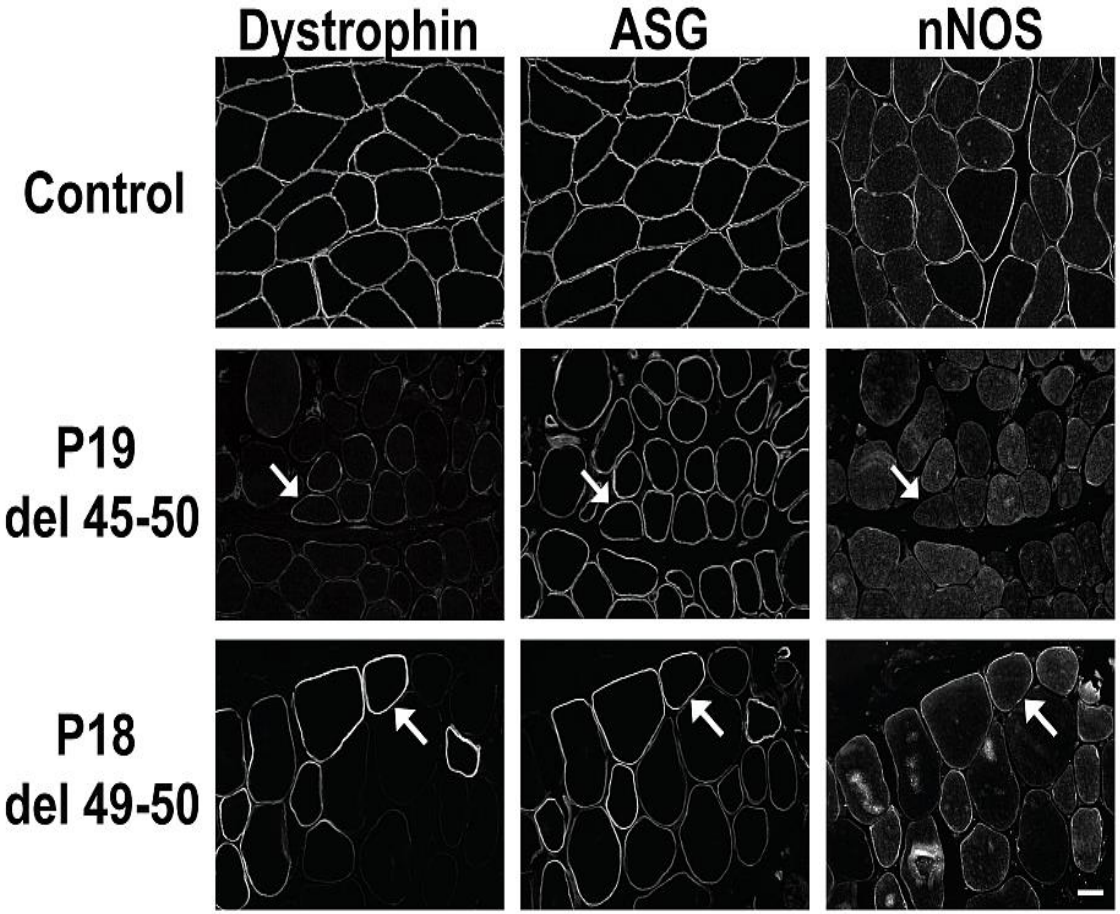
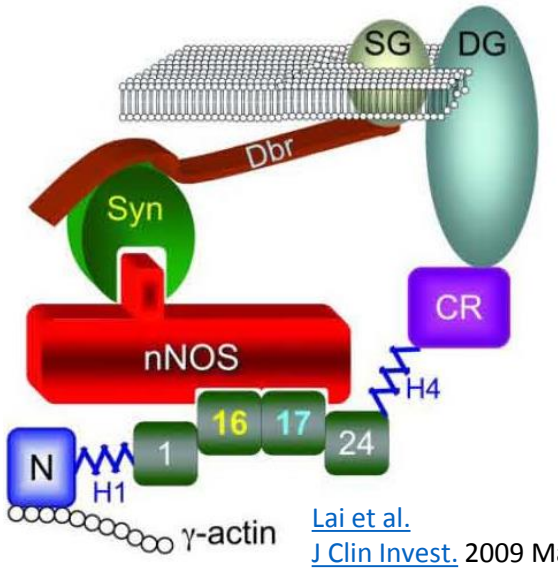
Functional proof of dystrophin presence

Indeed the novel dystrophin after exon skipping treatment with Eteplirsen restores the DGC and deletion specific nNOS.





Lai et al.
J Clin Invest. 2009 Mar

nNOS binding sites in dystrophin:
Exon 42-45,
Spectrin repeats 16-17

Cirak et al., 2011

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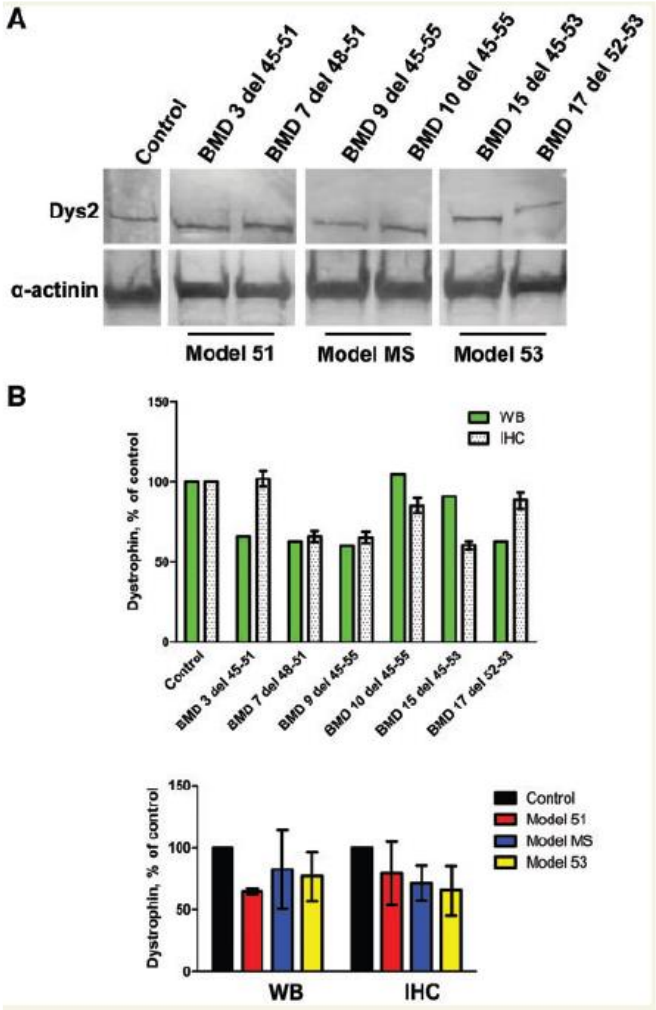
Cohort. Subject	Dys. positive fibres (%)		Mean Fluorescence Intensity / fibre (%)			Western-Blotting		Response to AVI-4658
	Pre	Post	Pre % of control	Post % of control	Increase %	Pre % of control	Post % of control	
1.P1	1	1	5	8	57	None	None	+
1.P2	3	0	5	5	0	None	None	+
1.P3	1	7	5	5	0	None	None	+
2.P5	0	0	4	4	0	None	None	+
2.P6	5	1	8	6	0	Trace	Trace	+
3.P7	1	21	5	19	314	2	18	+++
3.P8	1	5	7	5	-	None	None	+
4.P10	5	4	9	10	13	None	None	+
4.P11	1	1	8	11	30	1.1	0.7	+
5.P12	3	6	9	17	87	None	7	++
5.P13	2	6	11	10	-	None	9.6	++
5.P14	0	7	10	13	30	Trace	Trace	+
5.P15	1	15	9	27	198	0.9	17	+++
6.P16	3	5	11	13	16	0.5	None	+
6.P17	3	8	9	10	16	0.7	2.6	++
6.P18	3	55	9	19	110	None	7.7	+++
6.P19	5	7	10	13	24	5	12.3	++

Cochran-Armitage method and confirmed a significant linear trend of dose response leading to increase in dystrophin expression (responders with ++ or +++) with increasing dose (p=0.0203).

Dystrophin methods



doi:10.1093/brain/awr291 Brain 2011: 134; 3544–3556 | 3544

BRAIN
A JOURNAL OF NEUROLOGY

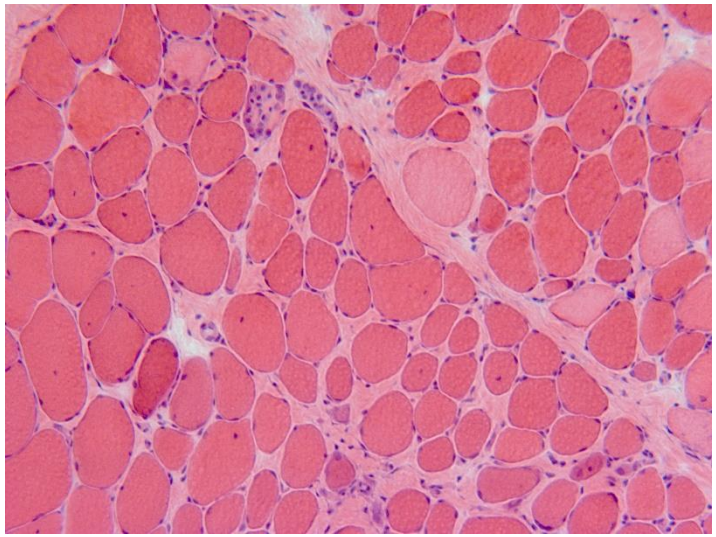
Dystrophin quantification and clinical correlations in Becker muscular dystrophy: implications for clinical trials

Karen Anthony,[1,*] Sebahattin Cirak,[1,*] Silvia Torelli,[1] Giorgio Tasca,[2] Lucy Feng,[1] Virginia Arechavala-Gomeza,[1] Annarita Armaroli,[3] Michela Guglieri,[4] Chiara S. Straathof,[5] Jan J. Verschuuren,[5] Annemieke Aartsma-Rus,[6] Paula Helderman-van den Enden,[6] Katherine Bushby,[4] Volker Straub,[4] Caroline Sewry,[1] Alessandra Ferlini,[3] Enzo Ricci,[7] Jennifer E. Morgan[1] and Francesco Muntoni[1]

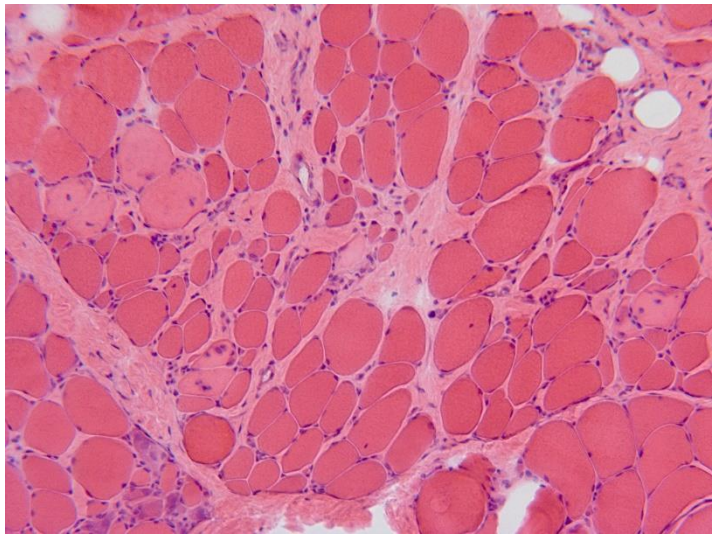
40 µg of protein loaded, good correlation between Western blot and immunofluorescence if the same antibody is used.

Solutions and Challenges

- Tissue heterogeneity in DMD muscle fat, connective tissue and myofibers
- Choice of muscle relevant: musculus biceps brachii is a good choice.



P18



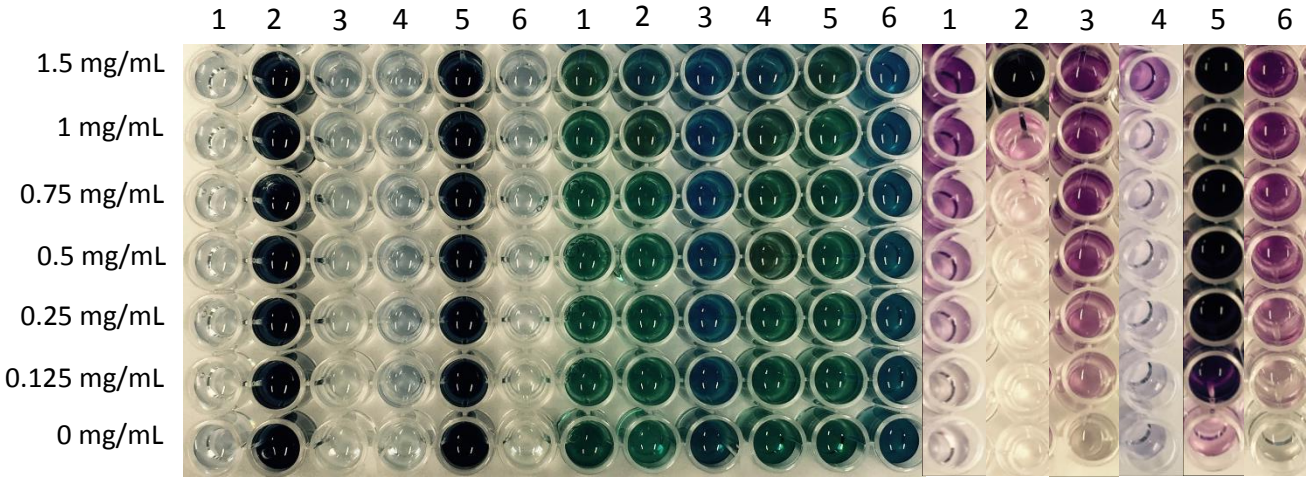
P19

Dystrophin can be detected if present at levels of even severe BMD patients with current methods.

Further standardisation required among lab also for the extraction procedures and buffer.

Lu et al., 2014 Mol Ther Nucleic Acids.

Protein quantification: Influence of different extraction buffers



BioRad RC DC Protein assay (Lowry based) | Bradford Assay | BCA protein quantification assay, Thermo Scientific

		R^2		
		Lowry	Bradford	BCA
Lysis buffers	1	0,4294	0,0152	0,9915
	2	0,7744	0,8204	0,9365
	3	0,9075	0,9225	0,9846
	4	0,3208	0,8686	0,8366
	5	0,9433	0,4256	0,7272
	6	0,9448	0,9058	0,9367

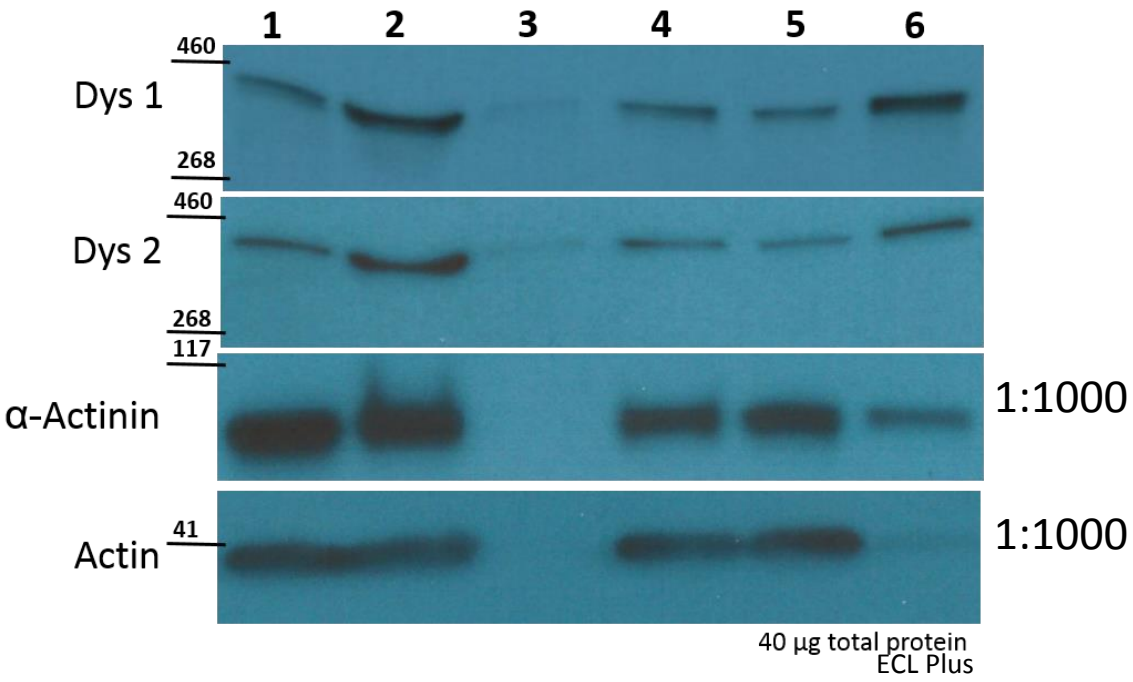
1. van Putten et al., 2013: **25% SDS**
2. Andersen et al., 1999: **4% SDS, 4 M Urea, 10% β-ME**
3. Wein et al., 2014: **1% NP-40, digitonin**
4. Cirak et al., 2011: **1% SDS**
5. Anthony et al., 2014: **9% SDS, 5% β-ME**
6. RIPA: **1% NP40, 0.1 % SDS, 0.5% Na-deoxycholate**

Extraction buffer comparison

Gastrocnemius of mouse

Cirak Lab Western Method:

- NuPage 3-8% Tris-Acetate gel
40 µg total protein loaded
25 V for 2 h on ice
- Wet blot: 10 V for 16 h @ 4°C
GE Amersham HyBond PVDF membrane
1° AB in 5% MP in TBST for 1 h @RT:NCL-Dys1 and Dys2 1:200, Sigma A7732 α-Actinin 1:1000
- 2° AB in TBST for 1 h @ RT
Pierce Goat-anti-mouse IgG, Peroxidase conjugated 1:2000



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6. RIPA: **1% NP40, 0.1 % SDS, 0.5% Na-deoxycholate**

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Thank you…

- **London, UK/Muntoni Lab:**

Francesco Muntoni, Lucy Feng, Virginia Arechavala-Gomeza, Karen Anthony, Silvia Torelli

- **Cologne, Germany/Cirak Lab:**

Lena Willkomm, Nicolas Berger

Emmy Noether-Programm

DFG Deutsche Forschungsgemeinschaft

CURE CMD

Congenital Muscular Dystrophy

SAREPTA THERAPEUTICS

MDA

Fighting Muscle Disease

Muscular Dystrophy Association • mda.org

LGMD2iFund

Limb Girdle Muscular Dystrophy 2I Research Fund

Disclosures

I was clinical investigator for following completed clinical trials:

- Phase 2b Study of PTC124 in Duchenne/Becker Muscular Dystrophy (DMD/BMD), NCT00592553
- Study of AVI-4658 to Induce Dystrophin Expression in Selected Duchenne Muscular Dystrophy (DMD) Patients, NCT00844597
- Safety and Efficacy Study of Antisense Oligonucleotides in Duchenne Muscular Dystrophy, NCT00159250
- My Laboratory is funded by the Germany Research Foundation (DFG) and the University Hospital Cologne/Germany.
- I have a sponsored research agreement with Sarepta Therapeutics.